1-1-2003

# Identification of other components in the ACR4 signal transduction pathway in Arabidopsis thaliana

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# Identification of other components in the ACR4 signal transduction pathway in *Arabidopsis thaliana*

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Molecular, Cellular, and Developmental Biology

Program of Study Committee:
Phil Becraft, Major Professor
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Iowa State University

Ames, Iowa

2003

Graduate College
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# ABSTRACT

The maize *CRINKLY4* (*CR4*) gene encodes a receptor-like serine-threonine kinase that is involved in an array of developmental processes, including cell differentiation, cell proliferation, cell fate determination and pattern formation. ACR4 contains all the features of maize CR4 and is believed to be the *Arabidopsis* ortholog. Yeast two-hybrid screening was used to isolate putative downstream targets of ACR4. Six proteins that interact with the cytoplasmic domain of ACR4 were identified, including a putative lipase, AJH1, AJH2, a protein phosphatase 2A (PP2A) regulatory subunit B delta, and two leucine-rich-repeat receptor-like kinases (LRR-RLKs). The interactions between these proteins and ACR4 were confirmed by in vitro pull-down assays. Substitution of a conserved $Lys_{540}$ with Ala in the kinase domain of ACR4, that abolished ACR4 kinase activity, did not affect the interactions, indicating that ACR4 kinase activity was not required for these interactions. *In vitro* kinase assays showed that ACR4 could phosphorylate AJH1, AJH2 and the two RLKs, but not the lipase or PP2A regulatory subunit B delta. Finally, the yeast two-hybrid system was used to show that the carboxyl-terminal domain is required for the interactions between ACR4 and the lipase, AJH1 and the PP2A regulatory subunit B delta, while AJH2, and both RLKs could interact with either the kinase domain or the C-terminal domain.

# CHAPTER 1. INTRODUCTION

The ability to perceive signals through cell-surface receptors is a common feature among all living organisms. In animals, the family of receptor tyrosine kinases (RTKs) mediates many signaling events at the cell surface. This class of receptors is defined structurally by the presence of a ligand-binding extracellular domain, a single membrane-spanning domain, and a cytoplasmic tyrosine kinase domain. The mechanisms by which most RTKs transmit signals are now well established. Binding of a ligand results in the dimerization of receptor monomers followed by transphosphorylation of tyrosine residues within the cytoplasmic domains of the receptors. In plants, receptor-like protein kinases (RLKs) have topological features of the RTKs but contain sequence motifs characteristic of serine/threonine kinases. RLKs belong to one of the largest gene families in the *Arabidopsis* genome, with at least 417 members that represent nearly 1.5% of *Arabidopsis* protein coding genes [1]. Recent analysis of kinases revealed that all the plant RLKs have a monophyletic origin in a group that contains Pelle, animal homologs of the RLK family, when compared to other eukaryotic serine/threonine/tyrosine protein kinases (ePKs) [1]. This group also includes receptor-like cytoplasmic kinases (RLCKs) that have no apparent signal peptide and transmembrane domain.

Plant RLKs are classified according to the amino acid sequence motifs in the putative extracellular domains. One of the largest and well-studied classes of RLKs is characterized by the leucine-rich repeat (LRR) motif, which is present in over half the *Arabidopsis* RLKs [1 and 2]. LRRs are known to be involved in protein-protein interaction [3 and 4]. In animals, LRRs are identified in many membrane proteins such as *Drosophila* Toll, but none of these proteins identified to date is a receptor protein kinase. The LRR-RLKs can be divided into 13 subfamilies based on the kinase domain amino acid sequence. Members of each subfamily have different numbers and arrangement of LRRs in their extracellular domains [1 and 2]. Many of the well-studied RLKs belong to the LRR-RLKs, including CLV1, HASEA, ERECTA, BRI1 and FLS2 [5, 6, 7, 8, 9]. It seems that the LRR domain of Plant RLKs may function in binding to the ligand.

The second largest class of extracellular motifs contains various sugar-binding motifs or lectins. This class includes 42 members of the lectin receptor protein kinases (LecRKs) with their extracellular domains similar to legume lectin originally found in the seeds of leguminous plants [2, 10 and 11]. Structural analysis of LecRKs and amino acid substitution in the putative monosaccharide-binding site suggest that the lectin domain is unlikely to be involved in binding monosaccharides as does true legume lectin, but can interact with complex glycans and/or with hydrophobic ligands [11 and

12]. Another type of lectin motif found in plant RLKs is the B-lectin, or agglutinin motif that is present in the N-terminus of the extracellular domain of 40 RLKs containing the cysteine-rich S-domain [2 and 12]. This group contains the family of S-locus receptor kinases (SRKs) in *Brassica*. They have an extracellular S-domain with high similarity to S-locus glycoproteins (SLGs) and are involved in the pollen self-incompatibility response [13]. SRKs also have a PAN module following the S-domain [2 and 12]. PAN modules are involved in protein-protein or protein-carbohydrate interactions and are found in several animal receptors, but not RPKs [12 and 14]. In addition to the lectin-type motifs, other types of carbohydrate-binding motifs are also present in plant RLKs. One of them is the lysin motif originally found in enzymes that degrade bacterial cell walls. It is also present in several other proteins involved in bacterial pathogenesis. Some evidence suggests that the lysin motif is a general peptidoglycan-binding module [2 and 15]. The chitinase-like motif found in tobacco chitinase-related receptor-like kinase (CHRK1) is another example of sugar-binding motifs. But the chitinase-like domain of CHRK1 lacks the essential glutamic acid residue and does not show any catalytic activity for either oligomeric or polymeric chitin substrates [2 and 16]. Other types include the thaumatin motif in PR5K, which is involved in binding to fungal cell walls [2 and 17] and the extensin motif that might interact with cell walls [12].

In addition to the LRR motif and the sugar-binding motifs mentioned above, there are several other types of motifs present in the putative extracelluar domains of RLKs. Wall associated kinases (WAKs) contain several epidermal growth factor repeats (EGF) and also other motifs such as collagen, neurexin, and tenascin in the extracellular domain [12, 18 and 19]. The EGF repeat is the only motif found in both plant RLKs and animal RTKs so far. The DUF26 motif, also called cysteine-rich repeats (CRRs), is found in at least 42 *Arabidopsis* RLKs (named CRKs for CRR RLKs). These RLKs contain four conserved cysteines and two C-X8-C-X2-C motifs in their extracelluar domains [12 and 20]. The CRINKLY4 family contains the tumor necrosis factor receptor (TNFR) like motif [21] and a putative RCC1 propeller domain [22]. Several others, such as proline-rich motif [12], CrRLK1-like motif [12 and 23] and LRK10-like motif [12 and 24], are also present in the plant RLKs with unknown functions.

Plant RLKs have been implicated in a diverse range of signaling processes, such as pollen development in *Petunia* (PRK1, [25 and 26]), meristem development (CLV1, [27]), the regulation of organ shape (ERECTA, [7]), brassinosteroid signaling (BRI1, [28]), the regulation of organ abscission (HASEA, [6]), self-incompatibility (SRKs, [29]), cell morphogenesis and differentiation (CR4, [21]; WAKs, [30]), plant-microbe interactions and stress response (Xa21, [31];

LRK10, [32]; FLS2, [9]), and somatic embrogenesis (SERK, [33]).

To fully understand the signaling cascade mediated by an RLK, it is necessary to identify other components (its ligands(s) and downstream substrate(s)) in the system. Putative ligands for SRK [SP11/SCR, 34], BRI1 [brassinolide, 35], LePRK [LAT52, 36] and FLS2 [flagellin, 37] have recently been identified. Other components of RLK signal transduction systems have also been found by a combination of genetic and biochemical approaches. KAPP, a type 2C protein phosphatase, has been shown to interact with CLV1 [38], and with several other RLKs [37, 39, 40 and 41]. Other examples include: CLV2 and ROP, which are part of the active CLV1 complex [42] and the *WUS* and *POL* genes, which are two targets of the CLV signaling system [43 and 44]; SLG appears to be involved in the self-incompatibility response and three other *Brassica* proteins, ARC1 and two thioredoxins that interact with SRK [45, 46, 47, 48, 49 and 50]; BAK1 appears to be a critical component of the brassinosteroid receptor complex [51 and 52] and BIN2, BRS1, BES1, BZR1 and TRIP-1 seem to be other components of BR signaling [53, 54, 55, 56 and 57]; KIP1 and a putative eIF2B β-subunit interacts with PRK1 of *Petunia inflata* [58 and 59]; a cysteine-rich extracellular protein, LAT52 interacts with tomato LePRK2 [60]; AGB1 is a possible component of the ERECTA signal transduction pathway [61].

The maize (*Zea mays*) *CRINKLY4* (*CR4*) gene encodes a receptor-like kinase that controls a variety of cell differentiation responses, particularly in the leaf epidermis and in the aleurone of the endosperm [21]. Mutants in the maize *cr4* gene disrupt aleurone differentiation, causing a mosaic aleurone phenotype and the defects are much more prevalent on the abgerminal face of the kernel. The mutants also have plant phenotypes with stunted plants due to shortened internodes and crinkled leaves. The leaf epidermis shows particularly strong effects on cell morphology and proliferation including abnormally large and irregular shaped epidermal cells, and the fusion of some regions. However, the internal leaf cells such as the mesophyll and vascular bundle cells appear less affected [62].

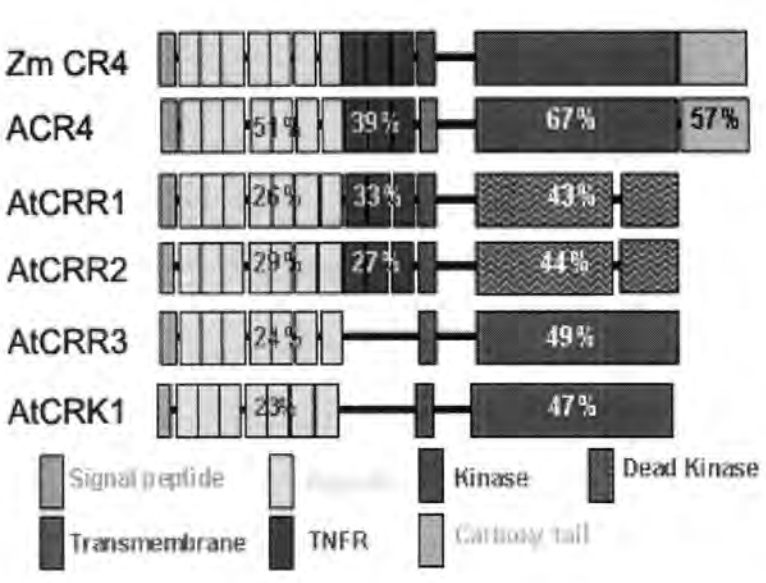
The *CR4* gene was isolated by transposon-tagging and the cDNA sequence revealed a 901-amino acid open reading frame. The maize CR4 gene encodes a novel class of RLK. The extracellular domain contains a cysteine-rich region that resembles the extracellular domain of mammalian tumor necrosis factor receptors (TNFRs), suggesting the ligand for CR4 may be a peptide related to tumor necrosis factor [62]. A second motif, consisting of a 39-amino acid repeat present in seven copies, may form an RCC1-like propeller structure, another protein interaction motif [22]. The presumptive cytoplasmic domain contains three motifs: an approximately 40-amino acid juxtamembrane region, a serine/threonine kinase domain and a

116-amino acid carboxyl-terminal domain with unknown function. The CR4 kinase domain, when expressed as a GST fusion protein in *E.coli*, autophosphorylates exclusively on serine and threonine residues. The activity is abolished by site-directed mutagenesis of the invariant $Asp^{652}$ to Ala, indicating that CR4 contains a functional serine/threonine protein kinase [62]. The CR4 gene contains no introns in the coding region, only a small intron in the 5′ UTR [63].

The *Arabidopsis* genome appears to encode five *CR4* related genes (Fig. 1). One, which is named *ACR4*, contains all the features of the maize CR4 and is believed to be the *Arabidopsis* ortholog. ACR4 protein shows 60% amino acid identity and 74% similarity with the maize CR4. The other four genes are designated as *AtCRR* (*Arabidopsis thaliana CRINKLY4-RELATED*) genes. AtCRR4 turned out to be similar to CRK1 in tobacco and was renamed AtCRK1 [64]. The overall amino acid conservation between AtCRRs and maize CR4 ranges from 31-32% identity and 46-48% similarity. All the four AtCRR proteins lack the carboxyl-terminal domain. AtCRR1 and AtCRR2 contain similar extracellular domains to maize CR4 and ACR4, but the kinase domain contains a deletion in subdomain VIII that is predicted to eliminate kinase activity [65]. AtCRR3 and AtCRK1 lack the TNFR-like repeats but have an intact kinase domain that is predicted to be functional.

CR4 signaling regulates an array of developmental processes in maize including cell fate, cell patterning, cell proliferation and differentiation suggesting a function analogous to growth factor responses in animals [62]. Several other mutants, including *dek1*, have similar phenotypes in both the endosperm and in the plant suggesting they may also function in the CR4 signaling pathway. Until now, little is known about the other upstream and downstream components in the CR4 signal transduction pathway. Differential screening and microarray analysis are currently being used to identify genes that are regulated by CR4 signaling. The identification of other signal molecules and components will lead to a better understanding of receptor kinase mediated signal transduction pathways, and of plant cell differentiation and communication. To study the CR4 signaling system in both maize and *Arabidopsis* will allow a comparison of the developmental functions of CR4 in monocots and dicots and will open up a wide range of possibilities for experiments using transgenic approaches.

In this thesis, we describe the isolation of six proteins that interact with the cytoplasmic domain of ACR4 using yeast two-hybrid screening. They are a putative lipase, AJH1, AJH2, a protein phosphatase 2A regulatory subunit B delta and two LRR-RLKs. Further characterization of the interactions suggest that the identified proteins bind ACR4 *in vitro* and might participate and play some roles in the ACR4 signal transduction pathway in *Arabidopsis thaliana*.




| | Overall % identity/similarity | ID |
|---|---|---|
| ACR4 | 60/74 | CAB91612 |
| AtCRR1 | 32/46 | AAF23257 |
| AtCRR2 | 31/48 | AAC28989 |
| AtCRR3 | 32/48 | CAB87849 |
| AtCRK1 | 32/47 | BAB11332 |

**Figure 1.** The *Arabidopsis* genome contains genes for at least 5 RLKs related to CR4. ACR4 contains all the motifs present in the maize CR4. AtCRR1 and AtCRR2 contain all the extracellular motifs of CR4. The cytoplasmic domains lack the carboxyl-terminal domains and the kinase domains appear to be non-functional. Both contain deletions that remove the essential residues in subdomain VIII. AtCRR3 and AtCRK1 contain what appear to functional kinase domains but also lack the carboxyl-terminal domains. They are also missing the region of similarity to TNFR. None of the genes appear to contain introns within the coding region.

# CHAPTER 2. MATERIALS AND METHODS

## 2.1. Yeast two-hybrid library screening

pGBD-C1 [67], a GAL4 DNA-binding domain vector, was used to construct the bait plasmid. The cDNA fragment encoding the cytoplasmic domain of ACR4 was ligated to BamHI and PstI sites of pGBD-C1 to generate an in-frame fusion between the DNA-binding domain of GAL4 and the cytoplasmic domain of ACR4, including amino acids 458-895 (pGBD-CR4). An *Arabidopsis* λACT cDNA library from 3 day-old seedlings was obtained from the *Arabidopsis* Biological Resource Center (stock number CD4-22) of the Ohio State University and used as the prey in the yeast two hybrid screening. The library was converted to plasmid and the plasmid DNA prepared using the Qiagen plasmid Maxi kit.

Yeast strain YRG2 (Stratagene) was used as the host stain. YRG2 yeast cells carrying pGBD-CR4 bait plasmid were transformed subsequently with DNA from the library by using the library transformation & screening protocols described in Yeast Protocols Handbook from Clontech. Approximately 1 million transformants were plated on synthetic dextrose (SD) minimal medium supplemented with 5mM 3-amino-1,2,4-triazole (3-AT) and lacking Trp, Leu and His. Those that grew were subjected to the colony-lift β-galactosidase filter assay (Clontech Yeast Protocols Handbook) for *lacZ* expression. 96

colonies turned blue in the *lacZ* assay, and white colonies were eliminated as false positives. These 96 colonies were then restreaked onto fresh SD/-His/-Trp/-Leu media with 3-AT to test whether they maintain the correct phenotype and activate reporter gene. Two colonies that did not regrow on the selective media were discarded. ONPG (o-nitrophenyl β-D-galactopyranoside) liquid culture assay (Clontech Yeast Protocols Handbook) was also used to verify and quantify the positive interactions. The positive colonies were then streaked on the SD/-Leu plates with 0.5g/l 5-FAA (5-fluoroanthranilic acid) after subculture three times in liquid SD/-Leu media. Total yeast DNA was extracted from colonies growing on these plates and then electroporated into *E.coli* HB101 cells to rescue the library plasmid. To eliminate duplicates, the library plasmid DNA were extracted and subjected to XhoI digestion to sort the colonies into groups based on insert size. To verify positive interactions, the library plasmid was then retransformed back into yeast YRG2 strain in combination with pGBD-CR4 and plated on SD/-His/-Trp/-Leu supplemented with 5mM 3-AT.

## 2.2. DNA sequence analysis

The cDNA inserts of the positive colonies were sequenced at the DNA Sequencing & Synthesis Facility of The Iowa State University. DNA sequences were analyzed with the BLAST program

[68] at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu) and Functional Genomics of Plant Phosphorylation (http://plantsp.sdsc.edu). Cellular localizations were predicted using PSORT [69] (http://psort.nibb.ac.jp) and SignalP [70] (www.cbs.dtu.dk/services/SignalP).

## 2.3. Protein expression

The putative cytoplasmic domain of ACR4 ($Arg_{458}$-$Phe_{895}$) was cloned in pET-32a(+) vector (Novagen) in frame with Trx/6X His/S-Tag at the N-terminus and 6X His at the C-terminus and expressed in *E. coli*. Expression of this protein, ACR4K, was induced in 1mM IPTG (Sigma) and proteins were purified from the cell lysate using TALON purification kit (Clontech). As a control, the Trx/His/S-Tag protein was expressed and purified from the empty pET-32a(+) vector (PET) using the same procedure as above. A kinase-dead ACR4 (ACR4KM) was also expressed in *E. Coli* by substituting the essential $Lys_{540}$ with Ala. The mutation was introduced by PCR where an AAG to GCG codon change was incorporated by primer mismatch.

The six putative interacting AD proteins (lipase, AJH1, AJH2, protein phosphatase 2A regulatory subunit B delta, CIRK1 and CIRK2) were expressed in *E. Coli* as GST-fusion proteins. The

cDNA inserts from the two-hybrid prey plasmids were PCR (primers are shown in Table 1) cloned into pBluescript (Stratagene) with a c-myc epitope tag added to the carboxyl terminus. After sequencing, the fragments were cloned into pGEX-5X1 (Amersham Pharmacia Biotech) between EcoRI and SalI sites to create an in-frame fusion with the sequence of GST. 1mM IPTG was used to induce the expression of these GST-fusion proteins and the proteins were purified from the cell lysate using Glutathione Sepharose™ 4B (Amersham Bioscience).

The putative kinase domain ($Arg_{458}$-$Val_{778}$) of ACR4 (BD-KD) and carboxyl-terminal domain ($Asp_{779}$-$Phe_{895}$) of ACR4 (BD-CT) were cloned into pGBD-C1 vector [67]. BamHI and PstI sites were introduced into the fragments by PCR. The digested PCR fragments were then ligated to the BamHI and PstI sites on pGBD-C1 to create an in-frame fusion with the sequence of GAL4 DNA-binding domain. Interactions with the six candidate proteins were tested as described above.

## 2.4. Pull-down assay

Approximately 5µg ACR4K, ACR4KM, or PET (encoding the thioredoxin tag, S-tag and 6X His tag) purified protein was combined with approximately 5µg lipase, AJH1, AJH2, phosphatase, CIRK1 or CIRK2 purified protein in 200µl binding buffer (50mM Tris [pH 8.8], 300mM NaCl, 50mM sodium phosphate,

and DOC (1.5% for interactions with lipase, AJH1 and phosphatase, 7% for interactions with AJH2, CIRK1 and CIRK2). After 45 minutes rocking at room temperature, 10µl TALON Resin (Clontech) was added into each combined solution with continued rocking at room temperature for additional 45 minutes. TALON Resin beads were then pelleted by brief centrifugation and washed for 6 times with 200µl binding buffer. 20µl 1x SDS loading buffer was added to each reaction and the resuspended beads were boiled for 5 minutes. After brief centrifugation, the supernatants were loaded on 10% SDS-PAGE gel and transferred to nitrocellulose membrane. The membrane was immunoblotted with the anti-myc primary antibody (Invitrogen, 1:5,000 dilution) and then with the goat anti-mouse IgG secondary antibody conjugated with horseradish peroxidase (Pierce, 1:10,000). Finally, the membrane was developed with the ECL western blotting detection reagents and analysis system (Amersham Bioscience), and exposed to Kodak X-Omat X-ray film.

## 2.5. Kinase assay

ACR4K purified protein was coupled to NHS-activated Sepharose 4 Fast Flow (Pharmacia Biotech) according to the manufacturers recommended procedure. First, wash the sepharose beads with 15 medium volumes of cold 1mM HCl. Then, mix the washed medium and ACR4K in coupling buffer (0.2M $NaHCO_3$ and 0.5M NaCl) and

incubate at 4°C overnight. After coupling is completed, block the non-reacted groups on the medium by standing in blocking buffer (0.5M ethanolamine and 0.5M NaCl, pH8.3) at room temperature for 2 hours. To wash the medium, use 3 x 1 medium volumes wash buffer (0.1M Acetic acid and 0.5M NaCl, pH 4.0) followed by 3 x 1 medium volumes blocking buffer. This cycle was repeated 3-6 times. Approximately 3µg NHS-activated sepharose-bound ACR4K was combined with approximately 5µg lipase, AJH1, AJH2, phosphatase, CIRK1, CIRK2 or ACR4KM in 60µl kinase buffer (50mM Tris-HCl [pH 7.5], 5mM $MgCl_2$, and 5mM $MnCl_2$). 1µl $\gamma$-$^{32}$P-ATP was added to each combined solution and the reaction was incubated at room temperature for 45 minutes. After brief centrifugation, the supernatants were loaded on a 10% SDS-PAGE gel. 20µl 1x SDS loading buffer was added to the remaining beads and the resuspended beads were boiled for 5 minutes. After brief centrifugation, the supernatants from the pelleted beads were also loaded on a 10% SDS-PAGE gel. The gels were then stained with Coomassie blue, dried, and subjected to phosphoimaging (Molecular Dynamics, Model 400A) to detect the phosphoproteins.

## 2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from wild type Columbia by grinding tissues in liquid nitrogen and using TRIzol reagent

(Invitrogen). RNA was then treated with RQ1 RNase-Free DNase (Promega) and purified with RNeasy Mini Kit (Qiagen) according to the manufacturer's instruction.

RT-PCR was performed to examine the expression of the lipase, CIRK1 and CIRK2 in the shoot apical meristem (SAM) and flower buds. 3μg total RNA from each tissue was used to synthesize first-strand cDNA. Reverse transcription was performed using SuperScript™ First-Strand Synthesis System for RT-PCR Kit (Invitrogen) according to the manufacturer's instruction. The subsequent amplification of target cDNA was performed using gene specific primers (Table 1). The amplification conditions were: 94°C 2min, followed by 35 cycles of 96°C 30s, 60°C 45s and 72°C 1min, ending with 72°C 10min. PCR products were run in 0.8% agarose gel and visualized with ethidium bromide staining.

**Table 1.** Primers used for PCR

| **Primer** | **Sequence (5′-3′)** | **Strand** |
|---|---|---|
| lipase-5 | GCAGGAATTCGAAAGCTATGGGCTGACGACGGCAGA | Top |
| lipase-3 | GACGGTCGACTAAGATCCTCCTCAGAAATGAGCTTTTGCTCCAAGAGTTGTGATGGATGATG | Bottom |
| AJH1-5 | GCAGGAATTCGAAGGTTCCTCGTCAG | Top |
| AJH1-3 | GACGGTCGACTAAGATCCTCCTCAGAAATGAGCTTTTGCTCCGATGTAATCATGGGCTCT | Bottom |
| AJH2-5 | GCAGGAATTCAGCATTCTAACAGTAGACTCAC | Top |
| AJH2-3 | GACGGTCGACTAAGATCCTCCTCAGAAATGAGCTTTTGCTCATATGTAATCATAGGGTCTGG | Bottom |
| RLK1-5 | GCAGGAATTCGCAATGGGTAATAAAGGAATGAGCGAGTT | Top |
| RLK1-3 | GACGGTCGACTAAGATCCTCCTCAGAAATGAGCTTTTGCTCTCCTTCGTTTTGCCATCTTTGTA | Bottom |
| phos-5 | GCAGGAATTCAACAACAATCAAGGAGGAGGT | Top |
| phos-3 | GACGGTCGACTAAGATCCTCCTCAGAAATGAGCTTTTGCTCCTTCGCCATTGAAGCAACAATCTCCT | Bottom |
| RLK2-5 | GCAGGAATTCGGGAAAGAGATTGCTGTAAAGGT | Top |
| RLK2-3 | GACGGTCGACTAAGATCCTCCTCAGAAATGAGCTTTTGCTCCCGAGCTGTTGGCTGCAATA | Bottom |
| BD-KD-5 | CTGAGGATCCAGATTGAGGAATTGTAGGT | Top |
| BD-KD-3 | AGCCCTGCAGCATTGATGGTCTATCTTTC | Bottom |
| BD-CT-5 | GTCGGGATCCGATAAAGTGACAACAGCATTGGAA | Top |
| BD-CT-3 | CGCACTGCAGGAAATTATGATGCAAGAAC | Bottom |

# CHAPTER 3. RESULTS AND DISCUSSION

## 3.1. Identification of proteins that interact with the cytoplasmic domain of ACR4 in the yeast two-hybrid system

To identify possible downstream targets of the ACR4 signal transduction pathway, we used yeast two-hybrid screening. The region containing the putative cytoplasmic domain of ACR4 was cloned into the bait vector pGBD-C1 [67] to produce an in-frame fusion with the GAL4 DNA-binding domain (pGBD-CR4). This construct was used as bait to screen an *Arabidopsis* 3 day-old seedling cDNA library to isolate proteins that interact with the cytoplasmic domain of ACR4. The bait (BD) plasmid and the prey (AD) library were transformed sequentially into the YRG2 yeast strain. Interactions between the bait and prey proteins should result in the activation of the reporter genes, *HIS3* and *lacZ*. From screening approximately 1 million yeast transformants, 94 colonies grew on the SD/-Trp/-Leu/-His selective medium supplemented with 5mM 3-AT and turned blue in the colony-lift filter assay for *lacZ* expression. The relative strength of these interactions was measured by using the ONPG (o-nitrophenyl β-D-galactopyranoside) liquid culture assay for β-galactosidase activity.

These positive clones were sorted into groups by restriction enzyme digestion and the representatives are subjected to sequencing. Sequence analysis revealed that these represented 6 independent clones. The results of the quantitative ONPG assays for these 6 clones are shown in Table 2. The strongest

**Table 2.** Interactions between the cytoplasmic domain of ACR4 and the candidate downstream targets in the yeast two-hybrid system.

| Sample | PGBD-CR4 | | | |
|---|---|---|---|---|
| | -LT SD[a] | -LTH SD[b] | β-Gal Activity (ONPG)[c] | β-Gal Activity (X-gal)[d] |
| lipase | + | + | 26.14 ± 5.59 | blue |
| AJH1 | + | + | 0.45 ± 0.04 | blue |
| AJH2 | + | + | 10.17 ± 2.98 | blue |
| phosphatase | + | + | 4.51 ± 0.76 | blue |
| CIRK1 | + | + | 0.20 ± 0.03 | blue |
| CIRK2 | + | + | 0.67 ± 0.03 | blue |

GenBank accession numbers for the interacting proteins:
lipase (GenBank accession nos. **AY099599**);
AJH1 (GenBank accession nos. **AF087413**);
AJH2 (GenBank accession nos. **AF087412**);
protein phosphatase 2A regulatory subunit B delta (GenBank accession nos. **AY091037**);
CIRK1 (GenBank accession nos. **NM 113279**);
CIRK2 (GenBank accession nos. **NM 129261**).
pGBD-CR4: the cytoplasmic domain of ACR4.
[a]The yeast transformants were streaked on the SD medium lacking tryptophan (-Trp) and leucine (-Leu) to ensure that both AD and BD plasmids were transformed into the yeast cell.
[b]The yeast transformants were streaked on the SD medium lacking tryptophan (-Trp), leucine (-Leu) and histidine (-His) but supplemented with 5mM 3-amino-1,2,4-triazole (+3-AT) to show the interactions.
[c]β-galactosidase activity was measured in a liquid culture assay using ONPG (o-nitrophenyl β-D-galactopyranoside) as substrate. Values shown are the averages from three assays.
Note: β-galactosidase units =1,000 x $OD_{420,\ average}$ / (T x V x $OD_{600}$)
Where: T = elapsed time (in min) of incubation
V = 0.1ml x concentration factor*
* The concentration factor is 5.
[d]The expression of the reporter *lacZ* gene was tested in a colony-lift filter assay using X-gal as the β-galactosidase substrate.

interaction is a protein that shows homology to a putative lipase. The program iPSORT predicts that this lipase has a chloroplast transit peptide, indicating that it is likely located in the plastids. ACR4 was predicted to localize to plasma membrane and as previously shown, an ACR4-GFP fusion proteins localized to the cell surface [64 and 71]. *ACR4* transcripts were detected at the highest levels in the SAM and flower buds, at intermediate levels in developing siliques, and low levels in mature leaves and roots (Cao and Becraft, unpublished data). RT-PCR was also performed to examine whether this lipase is expressed in the SAM and flower buds (Fig. 2). The lipase transcript was not detected in either the SAM or flower buds where *ACR4* showed strongest expression. The different expression patterns and predicted localization to different cellular compartments suggest that the lipase is unlikely to interact with ACR4 *in vivo*.

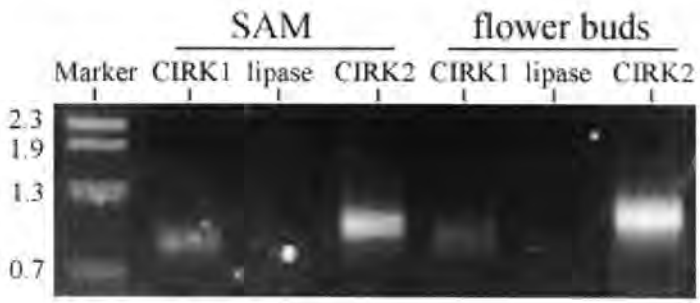



**Figure 2.** Expression of lipase, CIRK1 and CIRK2 in *Arabidopsis*. RT-PCR was used to examine the expression levels in SAM and flower buds. RT-PCR products were run on 0.8% agarose gel.
SAM: shoot apical meristem.

Two clones include AJH1 and AJH2 (*Arabidopsis* JAB1 homologs 1 and 2). *AJH1* and *AJH2* are two apparently redundant genes and were identified as subunits of the *Arabidopsis* COP9 complex [72]. AJH1 and AJH2 share 62% and 63%, respectively, amino acid identity to human JAB1 (c-Jun activation domain binding protein 1), a specific coactivator of AP-1 transcription [73]. JAB1 functions as an adaptor molecule in the formation of both nuclear and cytoplasmic protein complexes. Because of the high amino acid identity between the AJH proteins and human JAB1, it is possible that the AJH proteins may have a similar function as JAB1 and may be involved in several different pathways besides the COP9 complex. AJH proteins exist as both monomeric and complexed forms in *Arabidopsis* with the monomeric form predominantly in the cytoplasm and the complexed form predominantly in the nucleus [72]. AJH proteins are ubiquitously expressed in various *Arabidopsis* organs, including seedlings, siliques, flowers, leaves, stems and roots, with high expression in floral tissues and much lower expression in siliques and leaves [72]. As mentioned above, *ACR4* was expressed in various tissues with the strongest expression in SAM and flower buds. The overlapping expression patterns allow the possibility that AJH proteins participate in the ACR4 signal transduction pathway in *Arabidopsis*. The difference in the expression pattern is consistent with the AJH proteins functioning in other pathways, such as the COP9 complex.

The fourth clone shows homology to the type 2A serine/threonine protein phosphatase (PP2A) regulatory subunit B delta. In animal, PP2A proteins can exist as either heterodimers or heterotrimers. The heterodimer consists of the

catalytic subunit (PP2Ac) and the regulatory subunit A, whereas the heterotrimer contains an additional regulatory subunit B. Plants have homologs to all PP2A subunits. In *Arabidopsis*, there are at least five genes encoding the catalytic subunit, three genes encoding the A type regulatory subunit, two genes encoding the B type regulatory subunit, eight genes encoding the B′ type regulatory subunit, and one gene encoding the B′′ type regulatory subunit [74]. The clone we identified in the yeast two-hybrid screening belongs to the B′ type. These *Arabidopsis* PP2A genes are ubiquitously expressed although there are differences in the expression levels in different developmental stages indicating that PP2A might be involved in a diverse range of events [75]. Different combinations of the regulatory subunits and catalytic subunits determine the specificity, activity and subcellular localization of PP2A. It is unknown which type A regulatory subunit and catalytic subunit associate with the type B delta regulatory subunit identified in our two-hybrid screening. This candidate interacting protein is of interest because another phosphatase, KAPP (kinase-associated protein phosphatase), has been shown to interact with several RLKs *in vitro* and function as a negative regulator in CLV1 signal transduction pathway [44, 45, 46, 47 and 48]. KAPP is a type 2C protein phosphatase and *in vitro* studies suggest it could be a common component of more RLK systems. PP2A has never been reported to be involved in an RLK signal transduction pathway in plants. A possible role of PP2A in ACR4 signaling might also be as a negative regulator, similar to KAPP in other systems, by directly dephosphorylating ACR4 or another downstream component. Alternatively, the PP2A may act as a positive effector of ACR4 signaling, or function in a double

negative system where ACR4 inhibits PP2A which in turn inhibits downstream events.

Two clones encoding different LRR-RLKs (Leucine-rich repeat receptor-like kinase) were identified. LRRs often participate in protein-protein interaction [3]. CIRK1 and CIRK2 (CRINKLY4 interacting receptor kinase 1 and 2) contain 928 and 961 amino acids, respectively. The putative extracelluar domain of CIRK1 contains a signal peptide at the N-terminus, 5 LRRs followed by a glycine-rich domain, whereas that of CIRK2 contains only 2 LRRs (Fig. 3). Database searches revealed that CIRK1 is a member of LRRIX subfamily, whereas CIRK2 is a member of LRRI subfamily of Arabidopsis LRR-RLKs [1]. The cDNA inserts identified in the two-hybrid screening contain only the C-terminus of these two RLKs (most of the putative cytoplasmic domain, Fig. 3). This group of candidates is also of interest because they might form heterodimers with ACR4 in the receptor complex. Recent work has identified BAK1, an LRR-containing RLK, as a BRI1-interacting protein in a yeast two-hybrid screen using the cytoplasmic kinase domain of BRI1 as bait. It has also been shown that BRI1 and BAK1 form a heterodimeric receptor complex in the cell surface in *Arabidopsis* [51 and 52]. It is possible that the RLKs identified in our two-hybrid screening might also interact with the cytoplasmic domain of ACR4 *in vivo* and form heterodimers with ACR4. RT-PCR was performed to examine the expression of the *CIRK*s in the SAM and flower buds. As shown in Fig. 2, both *CIRK*s were expressed in these two tissues. The overlapping expression patterns leave open the possibility that the CIRKs might function in ACR4 signal transduction in *Arabidopsis*.

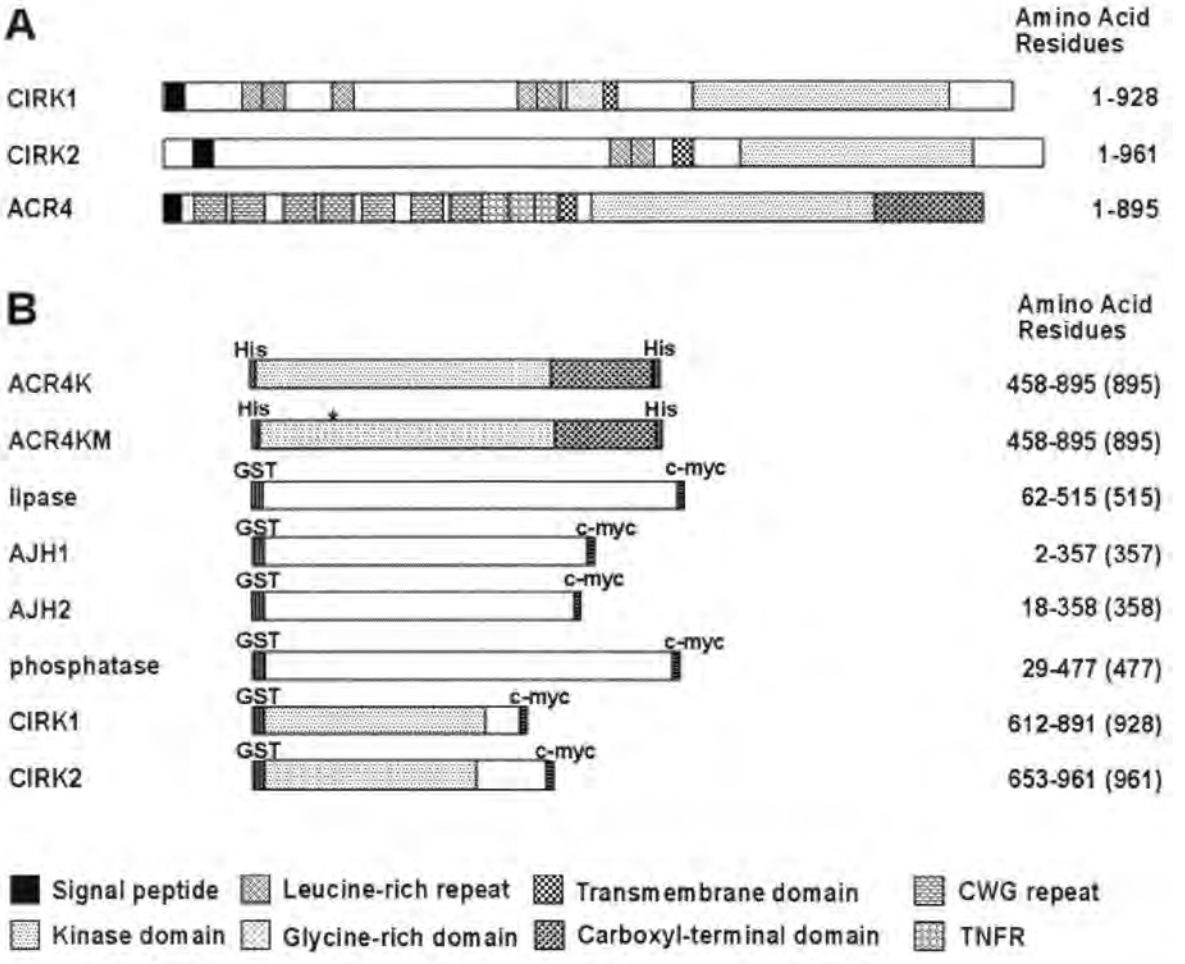



**Figure 3.** Schematic representation of the structural features of ACR4, CIRK1 and CIRK2 (A) and mapping of the domains of the AD proteins and ACR4 involved in the yeast two-hybrid screening (B). The drawings are to scale.
(A) The structures of the two CIRKs are predicted in the Functional Genomics of Plant Phosphorylation website (http://plantsp.sdsc.edu).
(B) For lipase, AJH1, AJH2, phosphatase, CIRK1 and CIRK2, GST denotes the glutathione S-transferase tag and c-myc denotes the 10-amino acid c-myc tag. For ACR4K and ACR4KM, His denotes the 6-amino acid His tag. An asterisk indicates the $Lys_{540}$ residue in the kinase domain of ACR4 that has been replaced with an Ala in ACR4KM. The first and last amino acid residues of each protein are indicated. The sizes of full-length proteins are shown in parentheses.

## 3.2. *In vitro* binding assay

To confirm the interactions between ACR4 and these identified proteins, an *in vitro* pull-down assay was carried out using *E.coli* expressed proteins. The cytoplasmic domain of ACR4 was cloned into pET-32a(+) expression vector to create a 6X His-tagged protein (ACR4K). The empty pET-32a(+) vector (PET), encoding the thioredoxin tag, S-tag and 6X His tag, was also expressed in *E. coli* and used as the control in the pull-down assay. The same regions of the interacting proteins as those identified in the yeast two-hybrid screening were cloned into pGEX-5X1 (Amersham Pharmacia Biotech) to make GST fusion proteins, and a c-myc tag was added to the C-terminus of each protein. The *in vitro* binding assay was performed by first incubating ACR4 and the interacting proteins together in binding buffer. ACR4K was then pulled down using TALON resin (Clontech) to bind the 6X His tag. Samples were separated by SDS-PAGE, blotted, and the presence of the interacting proteins detected using anti-myc antibody. As shown in Fig. 4, none of the fusion proteins interacts with the control Trx/6X His/S-Tag (PET) protein, while five of the six interacting proteins, including the lipase, AJH2, CIRK1, phosphatase and CIRK2, bind to the ACR4 cytoplasmic domain fusion protein. Only AJH1 did not show binding to ACR4K at a detectable level. This might be due to the poor expression of AJH1 protein in *E. coli*, as will be discussed in section 3.4 and 3.5. Thus, the lipase, AJH2, phosphatase and both CIRKs bind directly to the cytoplasmic domain of ACR4 *in vitro*.


**Figure 4.** The cytoplasmic domain of ACR4 interacts with the AD proteins in the pull-down assay and the interactions do not require ACR4 kinase activity. AJH2, CIRK1, CIRK2, AJH1, lipase or phosphatase were mixed with the empty pET-32a(+) vector (PET, lanes 1, 4, 7, 10, 13, and 16), the kinase-active ACR4 (ACR4K, lanes 2, 5, 8, 11, 14, and 17) or the kinase-inactive ACR4 (ACR4KM, 3, 6, 9, 12, 15, and 18) to examine the interactions. Lanes 1-3 contain the AJH2, lanes 4-6 contain the CIRK1, lanes 7-9 contain the CIRK2, lanes 10-12 contain the AJH1, lanes 13-15 contain the lipase, and lanes 16-18 contain the phosphatase. PET, ACR4K or ACR4KM was pull-downed by TALON resin, and the AD proteins were detected by anti-myc antibody.

## 3.3. The interactions between ACR4 and AD proteins do not require ACR4 kinase activity

Ligand binding typically induces phosphorylation of the cytoplasmic domain of receptor kinases, and interactions with downstream factors are often phosphorylation dependent. In this way, signal transduction components are recruited to an active receptor complex. To test whether the interactions between the cytoplasmic domain of ACR4 and the AD proteins require ACR4 kinase activity, an *in vitro* binding assay using a kinase-dead ACR4 was carried out. The AAG codon for the essential $Lys_{540}$ of ACR4 kinase domain was replaced by GCG for

Ala to create an inactive kinase (ACR4KM). ACR4KM does not autophosphorylate, indicating that the substitution abolished kinase activity (Cao and Becraft, unpublished data). As shown in Fig. 4, all the interacting proteins, except for AJH1, were pulled down with the kinase-dead ACR4 almost at the same level as with the active ACR4. Thus, the interactions between ACR4 and the lipase, AJH2, PP2A, or the CIRKs do not require ACR4 kinase activity.

## 3.4. Phosphorylation of the candidate interacting proteins by ACR4

A common mechanism in the regulation of signal transduction systems is for receptor kinases to regulate downstream components through phosphorylation. Previous work has shown that ACR4 is an active serine/threonine kinase ([64], and Cao and Becraft, unpublished data). To test whether ACR4 can phosphorylate the interacting proteins, we conducted a phosphorylation assay. Because the sizes of these interacting proteins were similar to the two strong ACR4K autophosphorylation bands, we covalently bound ACR4K to NHS-activated sepharose. After blocking the remaining active sites on the sepharose, the interacting proteins were mixed with the ACR4K-coupled sepharose in the kinase buffer supplemented with $\gamma$-$^{32}$P-ATP. After incubation, the sepharose was spun down and the supernatants applied to an SDS-PAGE gel (Fig. 5A). The remaining pellets were resuspended in SDS loading buffer, boiled, and the supernatants containing the released interacting proteins loaded onto another SDS-PAGE gel (Fig. 5C). Because ACR4K was covalently bound to the sepharose, it

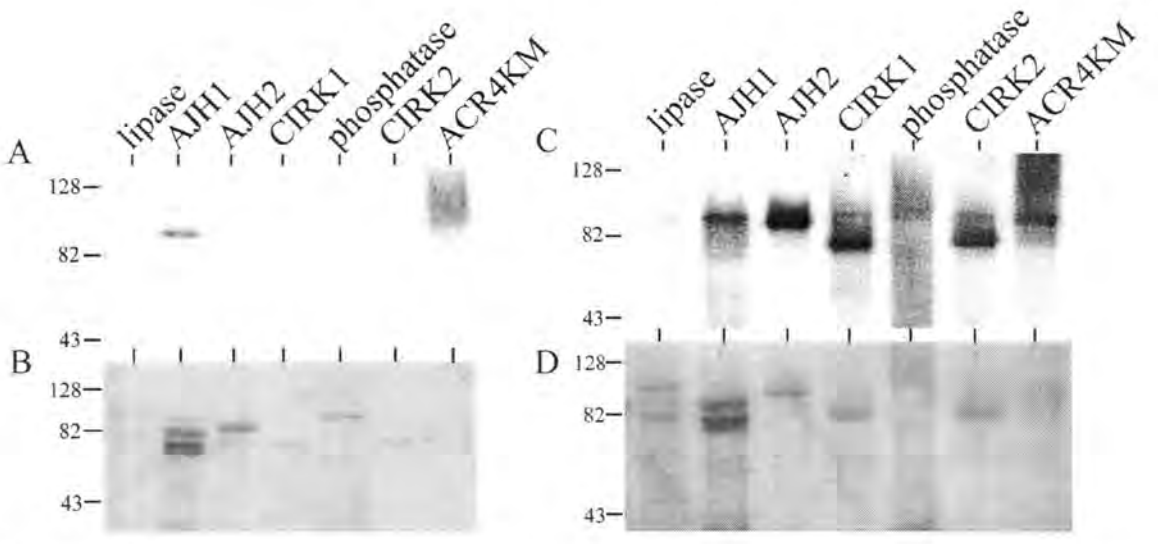



**Figure 5.** Phosphorylation of the AD proteins by ACR4. ACR4K-coupled sepharose was mixed with lipase, AJH1, AJH2, CIRK1, phosphatase, CIRK2 or the kinase-inactive ACR4 (ACR4KM) in kinase buffer while $\gamma$-$^{32}$P-ATP was added to each reaction. After incubated for 45 minutes, the sepharose was spun down and the supernatants were separated by a 10% SDS-PAGE (A). 20 µl 1X SDS loading buffer was added to the pellets of each reaction to resuspend the sepharose. After boiling for 5 minutes, the sepharose was spun down again and the supernatants from the pelleted sepharose was separated by a 10% SDS-PAGE (C). The Coomassie blue staining of the same gels in (A) and (C) was shown in (B) and (D).

remained bound with the sepharose and did not appear in either fraction.

The inactive kinase ACR4KM, which can be phosphorylated by the active kinase ACR4K (Cao and Becraft, unpublished data), was used as a substrate in the same kinase assay as a positive control. As shown in Fig. 5C, AJH1, AJH2 and both RLKs show a single clear phosphorylated band, whereas the lipase and phosphatase show little or no phosphorylation by ACR4K. To eliminate the possibility that the phosphorylation of the interacting proteins was caused by contamination in the protein preparation or by autophosphorylation, ACR4KM was used as a negative control in a similar kinase assay, instead of ACR4K. None of the interacting proteins showed phosphorylation by ACR4KM (data not shown), indicating that ACR4K phosphorylated the interacting proteins. Both CIRK clones contained only the C-terminus of the protein, including most of the kinase domain, but CIRK1 lacked subdomains I and II, and CIRK2 lacked subdomain I. That might eliminate their kinase activity, which is likely to be the reason we observed no autophosphorylation in the kinase assays. Thus, ACR4K can phosphorylate AJH1, AJH2 and both CIRKs *in vitro* indicating that they are *in vitro* substrates of ACR4.

Both the yeast two-hybrid results and the kinase assay indicate that the AJH1 protein interacts with the cytoplasmic domain of ACR4 *in vitro*, but this was not confirmed in the pull-down assay. This might be due to the poor expression of AJH1 protein in *E. coli*. The Coomassie blue stained gel (Fig. 6) showed three strong bands in the AJH1 lane. However, all three are smaller than the expected size. The phosphorylated

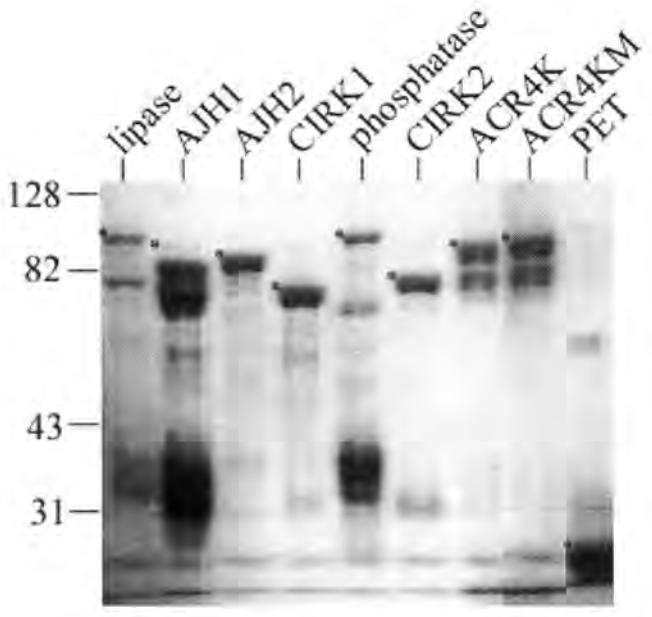



**Figure 6.** The expression of the AD proteins and the cytoplasmic domain of ACR4 in *E. coli*.
ACR4K: the cytoplasmic domain of ACR4.
ACR4KM: the cytoplasmic domain of ACR4 with the essential $Lys_{540}$ mutated to Ala.
PET: the pET-32a(+) expression vector.
The purified proteins were visualized by Coomassie blue staining. The asterisks mark the positions of the predicted full-length proteins.

AJH1 band in the phosphorylation assay was larger than any of these bands (Fig. 5) and was consistent with the predicted size of the protein comparing to the AJH2 band (Fig. 3 and Fig. 6). A weak band of the proper size is barely visible on the Coomassie gel (Fig. 5B, 5D and Fig. 6). If this weak band was the full-length AJH1 fusion protein with the smaller bands being the degradation products, then it appears that only the full-length AJH1 protein can interact with, and be phosphorylated by ACR4. The presence of phosphorylated AJH1 in the unbound fraction (Fig. 5A) also suggests a less stable

interaction, possibly contributing to the difficulty in detecting an interaction in pull-downs.

## 3.5. Binding to ACR4 is independent of the phosphorylation state of the interactors

Phospho-regulation of downstream factors is critical in many receptor kinase signal transduction systems. Phosphorylation might regulate activity, turnover, cellular translocation or further protein associations. As such, it is conceivable that phosphorylation of the interacting proteins could promote dissociation with ACR4. To test this, we compared the supernatants and pellet fractions from the phosphorylation assay (Fig. 5). While ACR4-bound proteins appeared in the pellet fraction, those proteins that no longer bound to ACR4 after phosphorylation should appear in the supernatants. As we can see in Fig. 5A, the phosphorylated AJH2 and CIRKs remained bound to ACR4 after phosphorylation, whereas part of the labeled AJH1 appeared in the supernatant after phosphorylation. This indicates that phosphorylation might cause some disruption of the protein-protein interaction, allowing part of the phosphorylated AJH1 proteins to dissociate from ACR4K. In contrast, phosphorylation of the other interactors did not destabilize the interaction with ACR4.

## 3.6. The carboxyl-terminal domain of ACR4 is required for the interactions between ACR4 and some of the interacting proteins

The cytoplasmic domain of ACR4 consists of three domains: the juxtamembrane region, the kinase domain, and a 116 amino acid carboxyl-terminal domain of unknown function. The complete cytoplasmic domain was used as bait in the two-hybrid screen. To test which domains are required for the interactions between ACR4 and the interacting proteins, we separately cloned the kinase domain (BD-KD) and the carboxyl-terminal domain (BD-CT) into the pGBD-C1 [67], a yeast two-hybrid bait vector. The prey plasmids containing the interacting clones were transformed into the YRG2 strain together with either BD-KD or BD-CT and the culture plated on the SD/-Trp/-Leu/-His medium supplemented with 5mM 3-AT to test for the *HIS3* reporter gene expression. The results are shown in Table 3: AJH2 and both CIRKs show interactions with either BD-KD or BD-CT, whereas lipase, PP2A, and AJH1 interact only with BD-CT but not with BD-KD in the yeast two-hybrid system. The interactions were confirmed by *lacZ* reporter gene expression in a colony-lift filter assay (Table 3). Thus, either the kinase domain or the carboxyl-terminal domain of the ACR4 is sufficient for the interactions between the ACR4 and AJH2 or the CIRKs, whereas the carboxyl-terminal domain of ACR4, but not the kinase domain, is necessary and sufficient, for the interactions between ACR4 and the lipase, PP2A or AJH1. Thus, the carboxyl-terminal domain of unknown function appears important for the interactions between ACR4 and potential downstream factors.

It is surprising that several proteins interacted with both the kinase and carboxyl-terminal domains. We searched for repeated motifs present in both the kinase and carboxyl-terminal domains that could be responsible for these

**Table 3.** Interactions between the AD proteins and the kinase domain of ACR4 or the carboxyl-terminal domain of ACR4 in the yeast two-hybrid system.

| | BD-KD | | | BD-CT | | |
|---|---|---|---|---|---|---|
| Sample | -LT SD[a] | -LTH SD[b] | β-Gal[c] | -LT SD[a] | -LTH SD[b] | β-Gal[c] |
| lipase | + | - | white | + | + | blue |
| AJH1 | + | - | white | + | + | blue |
| AJH2 | + | + | blue | + | + | blue |
| phosphatase | + | - | white | + | + | blue |
| CIRK1 | + | + | blue | + | + | blue |
| CIRK2 | + | + | blue | + | + | blue |

BD-KD: the kinase domain of ACR4.
BD-CT: the carboxyl-terminal domain of ACR4.
[a]The yeast transformants were streaked on the SD medium lacking tryptophan (-Trp) and leucine (-Leu) to ensure that both AD and BD plasmids were transformed into the yeast cell.
[b]The yeast transformants were streaked on the SD medium lacking tryptophan (-Trp), leucine (-Leu) and histidine (-His) but supplemented with 5mM 3-amino-1,2,4-triazole (+3-AT) to show the interactions.
[c]The expression of the reporter *lacZ* gene was tested in a colony-lift filter assay using X-gal as the β-galactosidase substrate.

interactions. As shown in Fig. 7, a potential motif was found in both domains. The motif consists of $SS(X)_9G(X)_6DE(X)_2K(X)_3A(X)_5EE(X)_3A$ (X stands for any amino acid) where the positions of SS, G, DE, K, A and EE are conserved. There is also an SEN before SS, and an SA between SS and G with their positions varied. This opens up the intriguing possibility that this repeated motif is responsible for the interactions with the proteins that recognized both the kinase and carboxyl-terminal domains of ACR4.

| | | |
|---|---|---|
| RLRNCRC**SEN**DTR**SS**KD**SA**FTKDN**G**KIRPDL**DE**LQ**K**RRR**A**RVFTY | 502 | Kinase |
| **EE**LEK**A**ADGFKEESIVGKGSFSCVYKGVLRDGTTVAVKRAIMSSD | 547 | domain |
| KQKNSNEFRTELDLLSRLNHAHLLSLLGYCEECGERLLVYEFMAH | 592 | |
| GSLHNHLHGKNKALKEQLDWVKRVTIAVQAARGIEYLHGYACPPV | 637 | |
| IHRDIKSSNILIDEEHNARVADFGLSLLGPVDSGSPLAELPAGTL | 682 | |
| GYLDPEYYRLHYLTTKSDVYSFGVLLLEILSGRKAIDMHYEEGNI | 727 | |
| VEWAVPLIKAGDINALLDPVLKHPSEIEALKRIVSVACKCVRMRG | 772 | |
| KDRPSM | 778 | |
| DKVTTALERALAQLMGNPSSEQPILPTEVVLGSSRMHKKSWRIGS | 823 | Carboxyl- |
| KRSG**SEN**TEFRGGSWITFPSVT**SS**QRRKS**SA**SE**G**DVAEEE**DE**GR**K** | 868 | terminal |
| QQE**A**LRSLE**EE**IGP**A**SPGQSLFLHHNF | 895 | domain |

**Figure 7.** A possible repeated motif, $SS(X)_9G(X)_6DE(X)_2K(X)_3A(X)_5EE(X)_3A$, is present in both the kinase and carboxyl-terminal domains. The putative cytoplasmic domain of ACR4, consisting of the kinase and carboxyl-terminal domains is shown above. The conserved amino acids are in bold. The amino acids with conserved position are double-underlined, whereas those with varied position are single-underlined.

# CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTIONS

## Conclusions

The maize *CRINKLY4* gene encodes a receptor-like kinase that is involved in an array of developmental processes in maize including cell fate, cell patterning, cell proliferation and differentiation. ACR4 is believed to be the *Arabidopsis* ortholog and contains all the features of maize CR4. Until now, little is known about the other upstream and downstream components in the CR4 signal transduction pathway.

In this thesis, we describe the isolation and characterization of proteins that might be the downstream targets in ACR4 signal transduction pathway. The yeast two-hybrid screening resulted in the identification of six proteins that interact with the cytoplasmic domain of ACR4, including a putative lipase, AJH1, AJH2, a protein phosphatase 2A regulatory subunit B delta, and two LRR-RLKs. The *in vitro* interactions between ACR4 and the interacting proteins were further confirmed by an independent pull-down assay. Our results showed that ACR4 could phosphorylate AJH1, AJH2, and the two RLKs *in vitro*. The interactions occurred independent of the phosphorylation state of the interacting proteins, or of CR4 kinase activity. The carboxyl-terminal domain of ACR4 is required for the interactions with some of the downstream targets. Though further investigations are required to examine the interactions *in vivo* and determine the possible functions of these interacting proteins, our results suggested that

these interacting proteins might be the downstream targets in the ACR4 signal transduction pathway.

## Future directions

Based on the results of the yeast two-hybrid screening and pull-down experiments, we will focus on one or two CRINKLY4 interacting proteins (CIPs) for further functional analysis. First, we will search the database of T-DNA insertions mapped by SIGnAL (Salk Institute Genomic Analysis Laboratory, http://signal.salk.edu/cgi-bin/tdnaexpress) for the knockout mutants of CIPs. If the knockouts are not available in SIGnAL, other resources, such as Wisconsin Arabidopsis knockout pools (http://www.biotech.wisc.edu/Arabidopsis/) and Sygenta Arabidsopsis Insertion Library or "SAIL" (formerly GARLIC, http://www.nadii.com/pages/collaborations/garlic_files/GarlicDescription.html), will be used to screen for the knockout mutants. Once the knockout mutants are available, we can check them for any phenotypic alteration. Plants with mutation in *acr4* don't show any obvious plant phenotype, which might be due to the existence of redundant function genes (or pathways), so the phenotype of the CIP knockouts might give us some ideas about how ACR4 signaling affects plant development. To identify the possible roles of those CIPs in ACR4 signaling, we can make transgenic plants that overexpress the CIP under the CaMV35S promoter and check for any phenotypic alteration. These transgenic lines might show opposite phenotypes to the loss-of-function mutants. Next is to examine the expression pattern of the CIPs by the northern blot analysis and *in situ* hybridization. GFP fusion CIPs can be constructed to check the subcellular localization of CIPs and

double-immunolabeling can be used to check whether ACR4 and CIPs colocalize in the same cells. If these CIPs function in the ACR4 pathway, we would expect that the expression domains of them should have some overlapping with that of ACR4. To further investigate possible association between ACR4 and the CIPs *in vivo*, coimmunoprecipitation can be performed when we have antibodies against both CIPs and ACR4 (the generation of anti-ACR4 antibody is in process). The results of coimmunoprecipitation will show us whether there are direct interaction between ACR4 and the CIPs *in vivo*. Phosphorylation/dephosphorylation is an important part of the signal transduction pathway. Previous results have shown that some CIPs can be phosphorylated by ACR4, so we can also test whether phosphorylation by ACR4 alters the activity of them. Further functional analysis will depend on the properties of the CIPs we have chosen. For example, for the phosphatase, we can investigate the interaction between ACR4 and phosphatase by testing the ability of phosphatase to dephosphorylate ACR4 *in vitro*; for the putative RLKs, we can perform kinase assays to see whether these RLKs can autophosphorylate and whether ACR4 and these RLKs can transphosphorylate each other, and so on.

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## ACKNOWLEDGEMENTS

I would like to thank Dr. Phil Becraft, my major professor, for all the guidance, suggestion and help to my research work and thesis writing. Also, thanks to all the members in Becraft's lab, Xueyuan Cao, Juan Liu, Antony Chettoor, Pei Wang and Nrisingha Dey, for all the help they gave me. Finally, I would like to thank Xiaotao Zhou, my husband and my parents for their support, advice and encouragement.